Quantitative Bio-Science 34(2), 73~77(2015)

Effects of Micro- and Nano-sized Iron Particles on Colonic Pre-neoplastic Lesions in an Experimental Mouse Model

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(Received November 5, 2015; Revised November 22, 2015; Accepted November 24, 2015)

ABSTRACT

Although iron (Fe) plays a crucial role in our body, iron-overload can cause harmful effects such as cancer and aging via promoting the production of free radicals. The effects of orally administered micro-Fe and nano-Fe on colon carcinogenesis were investigated in male ICR mice. After acclimation for 1 week, six-week old mice received 3 i.p. injections (0-2nd weeks of the experiment) of azoxymethane (AOM, 10 mg/kg b.w.), followed by 2% dextran sodium sulfate (DSS)-containing drinking water for the next 1 week to induce aberrant crypt foci (ACF). Animals were fed on AIN-76A purified rodent diet (34.25 Fe mg/kg diet) for 8 weeks. In addition, each group of animals was orally treated with saline alone, carboxymethylcellulose (CMC) + micro-Fe mineral or CMC + nano-Fe mineral daily for 8 weeks. The daily doses were 48 ppm iron for control group and 480 ppm iron for micro-Fe and nano-Fe groups. After the colonic mucosa were stained with methylene blue, aberrant crypt foci (ACF) and aberrant crypts (AC) were counted. Lipid peroxidation in serum and liver was evaluated by the thiobarbituric acid-reactive substances (TBARS) assay. The numbers of ACF and total AC per cm^2 of colon significantly increased in nano-Fe group (ACF: 15.74, AC: 23.44) compared to the control group (ACF: 8.69, AC: 16.60) or micro-Fe group (ACF: 11.20, AC: 17.10) ($p < 0.05$). TBARS values in serum and liver significantly increased in micro-Fe and nano-Fe groups compared with the control group ($p < 0.05$). The iron levels in the micro-Fe group and the nano-Fe group were significantly higher than the control group as determined by the ICP method ($p < 0.05$). These results suggest that additionally administrated micro-Fe and nano-Fe can affect the colon carcinogenesis in a mouse model. In addition, the nano-Fe may be more harmful than the micro-Fe in colon carcinogenesis.

Key words : Aberrant crypt foci, Azoxymethane, Dextran sodium sulfate, Colon cancer, Iron Nanoparticle

1. Introduction

According to Statistic Korea, the incidence of cancer increases constantly and the mortality rate per 100,000 population due to cancer is 149 in 2013 so the cancer is the leading cause of death [1]. Among other cancers, the mortality rate due to colon cancer has increased 16.4% in 2013 compared to 11.4% in 2003 and the colon cancer is the second leading cause of death from cancer in women [1]. Despite the medical advances, the mortality rate from colon cancer is still high. In this respect, many studies on colon cancer has been conducted actively. It is known that colon cancer is related with the excessive intake of red meat. Red meat consumption is thought to increase gut lumen concentration of iron, which can cause to produce reactive oxygen species (ROS) via the Fenton reaction [2]. ROS may interact with and modify cellular protein, lipid, and DNA, which results in altered target cell function. The Fenton chemistry leads to formation of hydroxyl radicals. The resultant oxidative stress leads to the extension and propagation of crypt

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abscesses, either through direct membrane disruption by lipid peroxidation or through generation of secondary toxic oxidants such as chloramines [3]. Oxidative DNA damage may participate in ROS-induced carcinogenesis [4]. Hydroxyl radicals are believed to promote tumor development by causing chromosomal damage. Thus, excess body iron stores and inappropriate iron administration may interfere with normal body defenses and promote cancer [5].

The initiation of a single epithelial cell within the crypt to undergo histogenetic changes and gradually proliferate to form morphologically distinct foci is the first and foremost stage in the genesis of aberrant crypt foci (ACF) in the colon [6]. Evidence is mounting supporting the idea that ACF are colon cancer precursors whose size and numbers directly correlate with the risk of developing colon cancer [7].

Mice treated with the carcinogen azoxymethane (AOM) can induce the growth of colonic crypts that are larger, thicker and darker [8]. The combination of a single hit of AOM with 1 week exposure to the inflammatory agent dextran sodium sulphate (DSS) in rodents has proven to dramatically shorten the latency time for induction of colon cancer [9].

Although there are a lot of studies to compare abilities to induce the pre-neoplastic lesions between excessive and deficient dietary iron, any study to compare the effects of micro-Fe and nano-Fe on the formation of colonic pre-neoplastic lesions was not conducted so far. The aims of this study were to elucidate the influence of the orally administrated micro-Fe and nano-Fe on the formation of colonic ACF induced by AOM + DSS in mice.

2. Materials and Methods

2.1 Experimental materials

Azoxymethane (AOM) was obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA). Dextran sodium sulfate (DSS) was obtained from MP Biomedical Inc. (USA). Micro-Fe (<5 μm, 95%) and nano-Fe (<50 nm, $\geq 98\%$) which are all powder forms were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA).

2.2 Animals and experimental diets

Male ICR mice (5 weeks old) were obtained from Central Laboratory Animal Inc. (Seoul, Korea) and housed in isolating polycarbonate cage (5 mice/cage). The temperature and relative humidity were maintained at 20 ± 2°C and 50 ± 20%. Light and dark cycles were at 12 h each and the intensity of illumination was maintained at 150-300 lux. The AIN-76A purified diet from Dyet Inc. (Bethlehem, USA) was given to the mice. During the experimental periods, diets and litter were all used after sterilization and the animal experiment was conducted in compliance with "Guide for care and use of Laboratory animals" of Chungbuk National University. During the experimental period, weekly body weight and feed consumption were recorded.

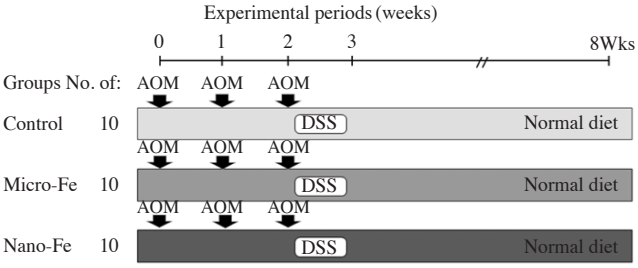

Fig. 1. Experimental design for treatment groups and induction of colon carcinogenesis in mice. AOM, azoxymethane (10 mg/kg body weight in saline, I.P., weekly 3 times). DSS, dextran sodium sulfate (2% in drinking water for a week). Micro-Fe; micro-iron (micro-Fe, 432 ppm Fe in distilled water. P.O., daily). Nano-Fe; nano-iron (nano-Fe, 432 ppm Fe in distilled water. P.O., daily)

2.3 Experimental design

After acclimation for 1 week, thirty six-week old mice was divided into 3 groups (10 mice/group) including (1) control group, (2) micro-Fe group, (3) nano-Fe group. AIN-76A purified diet (34.25 Fe mg/kg diet) and distilled water were fed to the three groups. Additionally, saline was orally administrated to the control group. Micro-Fe and nano-Fe (432 Fe mg/kg B.W.) were mixed with 1% carboxymethyl cellulose (CMC) so that they were total 480 mg/kg B.W. (48 ppm Fe diet + additionally 432 mg/kg B.W.) and they were orally administrated to the micro- and nano-Fe group each. Also the mice were subcutaneously treated with AOM (10 mg/kg) three times at 0, 1st and 2nd weeks of the experimental period to induce the formation of pre-neoplastic lesion in colon. From the next day, water containing 2% DSS was provided for 7 days and the total experimental period is 8 weeks (Fig. 1).

2.4 Sample collection

At 8 weeks after the experiment, all mice were sacrificed. After anesthesia and laparotomy, blood was collected by a syringe from the abdominal aorta. The liver, spleen, kidneys

and entire large intestine were harvested and then frozen in liquid nitrogen (−196°C). A half of the large intestine was washed with saline and fixed in 10% neutral buffered formalin and the other half was frozen in liquid nitrogen.

2.5 AC and ACF counts

The colon were harvested and flushed with 0.9% NaCl solution and weighted and finally fixed in 10% neutral phosphate buffered formalin. The formalin-fixed colonic tissues were stained in 0.5% methylene blue solution for 30 sec and the total number of ACF and the number of aberrant crypts (AC) in each focus were counted under a microscope (40-100x). The total number of ACF and the number of AC were marked as number/cm^2.

2.6 Thiobarbituric acid reactive substances (TBARS) assay

TBARS assay was conducted for determination of malondialdehyde (MDA) of which is a by-product of lipid peroxidation in the serum and liver. At first, collected sample 100 μL, 8.1% sodium dodecyl sulfate (SDS) solution 100 μL, 20% acetic acid solution 200 μL were mixed together and 0.75% 2-thibarbituric acid (TBA) solution 100 μL were added lastly and they were vortexed and spined down. Then, the incubation at 95°C was conducted for 30 min and the centrifuge process was conducted at 4°C, 13000 rpm for 15 min. At last, they were put in the 96-well plate and the optical density of the supernatant liquid was measured at 532 nm. The protein quantitative analysis was conducted with using DC Protein kit (Bio-rad Laboratories, Inc., Hercules, CA) which were applied by the Lowry method.

2.7 Iron analysis in liver

For the determination of total iron, samples of liver were analyzed by ICP-AES (Inductively coupled plasma spectrophotometer) (OPTIMA 7300DV, Perkin Elmer, USA). Frozen samples were digested and ashed at 200°C for 4 h using concentrated nitric acid and hydrogen peroxide. For the ICP-AES analysis of iron, the digested sample was diluted with equal amounts of de-ionized water before analysis.

2.8 Statistical analysis

Data were expressed as the means ± standard deviation (SD). Data were analyzed with the one-way ANOVA and the Tukey's test. The significant differences were determined at the level of $p<0.05$ or $p<0.01$.

3. Results

3.1 Changes in body weights

The mice body weights of all experimental groups were increased with the passage of time but the body weights of nano-Fe group were slightly low compared with control group (Fig. 2). Micro-Fe group had nearly same body weights with control group and slightly increased body weight compared with nano-Fe group. At 8th week of the experimental periods, nano-Fe group had low body weights compared with control and micro-Fe group and there was a significant difference compared with control group ($p<0.05$).

3.2 ACF and AC counts

The number of ACF/cm^2 and the number of AC/cm^2 in micro-Fe group (ACF: 11.20, AC: 17.10) were increased compared with control group (ACF: 8.69, AC: 16.60) but there was no significant differences (Fig. 3). The number of ACF/cm^2 and the number of AC/cm^2 in nano-Fe group was significantly higher than either control group or micro-Fe group ($p<0.05$).

3.3 TBARS values in serum and liver

In serum, the TBARS values of micro-Fe group (28.37 nM/mL serum) and nano-Fe group (34.05 nM/mL serum) were higher than control group (23.48 nM/mL) and especially nano-Fe group had significantly higher TBARS value compared

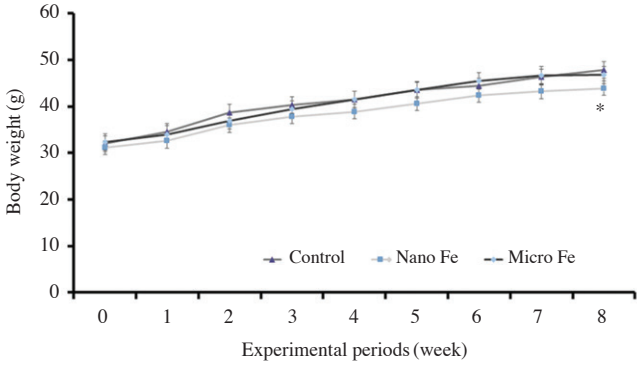

Fig. 2. Change in the body weights in mice treated with AOM/DSS, micro-Fe and nano-Fe. The body weights of all groups increased as time goes on. *Significantly low compared with the control group ($p<0.05$).

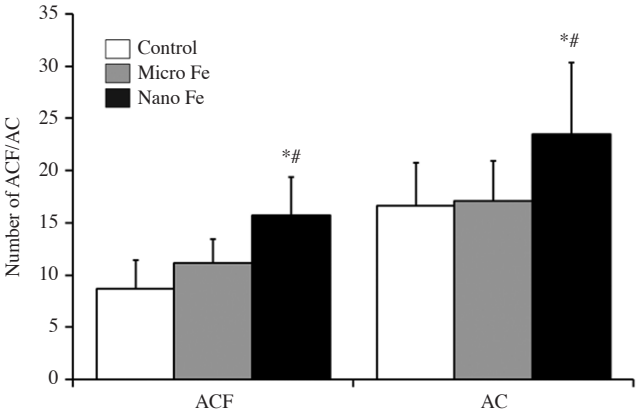

Fig. 3. Effect of orally administrated micro-iron (Fe) and nano-iron (Fe) on formation of colonic aberrant crypt foci (ACF) and total aberrant crypt (AC) in mice. Each bar means the mean ± SD. *Significantly different from control group ($p < 0.05$). #Significantly different from micro-Fe group ($p < 0.05$).

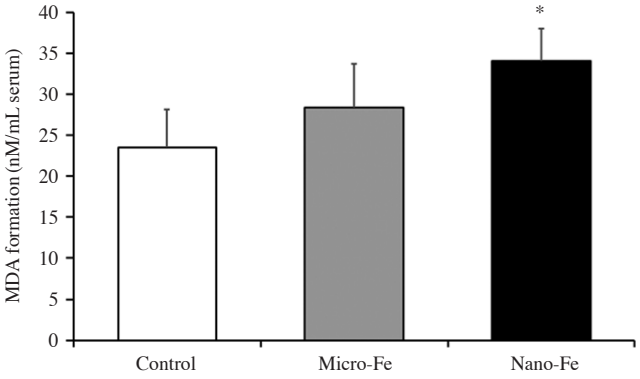

Fig. 4. Effect of micro-iron (Fe) and nano-iron (Fe) on lipid peroxidation in the serum measured by TBARS assay. Micro-Fe and nano-Fe group increased the MDA formation more compared to control group. Each bar means the mean ± SD. *Significantly different from control group ($p < 0.05$).

with control group ($p < 0.05$) (Fig. 4). In liver, the TBARS values of micro-Fe group (0.083 nM/mg protein) and nano-Fe group (0.091 nM/mg protein) were significantly higher than control group (0.071 nM/mg protein) ($p < 0.05$) but there was no significant differences between micro-Fe group and nano-Fe group (Fig. 5).

3.4 Iron concentration in liver

Iron store in the liver was measured by an inductively coupled plasma spectrophotometer (ICP-AES). The micro-Fe group (2.36 Fe mg/kg) and the nano-Fe group (2.79 Fe mg/kg) had higher deposits of iron in the liver of mice than the control group (1.33 Fe mg/kg) ($p < 0.05$) (Fig. 6).

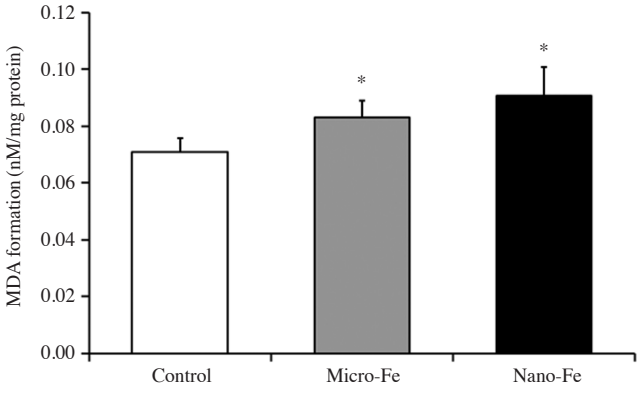

Fig. 5. Effect of micro-iron (Fe) and nano-iron (Fe) on lipid peroxidation in the liver measured by TBARS assay. Micro-Fe and nano-Fe group increased the MDA formation more compared to control group. Each bar means the mean ± SD. *Significantly different from control group ($p < 0.05$).

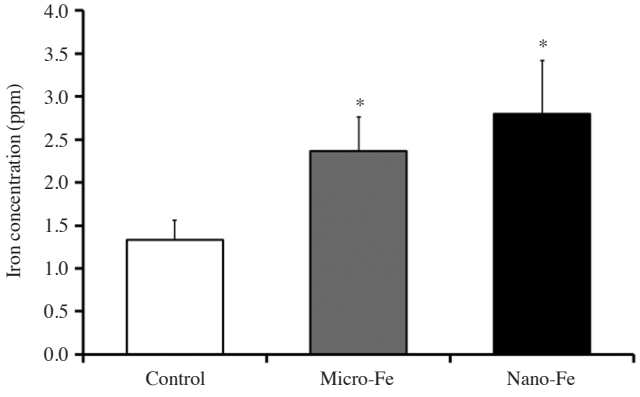

Fig. 6. Iron concentration in the liver of male ICR mice. Each bar means the mean ± SD (n = 10). *Significantly different from control group ($p < 0.05$).

4. Discussion

The objective of the present study was to determine a relation between the particle size of iron and colon carcinogenesis as evaluated by colonic ACF induced by AOM + DSS in male ICR mice. The mice were fed on AIN-76A purified diets containing 48 ppm Fe and additionally saline, micro-Fe (432 ppm Fe) and nano-Fe (432 ppm Fe) mixed with 1% carboxymethyl cellulose (CMC) were administrated to each mice daily.

In the present study, the number of ACF/cm^2 and the number of AC/cm^2 in the control, micro-Fe and nano-Fe group were counted. Bergeron et al. found that *in vivo* studies clearly indicate higher numbers of tumor cells and a shorter life span in the presence of supplemental iron [10]. Knobel et al. also concluded that high concentration of iron caused the formation

of ROS and carcinogenic risks from ferric iron enhanced tumor cell growth and cause progenotoxic effects in *in vitro* study [11]. In the present study, micro-Fe increased the total numbers of ACF as a pre-neoplastic lesion in the colon of mice although there was no a significant difference from the normal iron control. On the other hand, nano-Fe increased the total numbers of ACF in the colon of mice and there were significant differences compared with the control or the micro-Fe group. From these results, it seems that nano-Fe was absorbed to the body more than micro-Fe, resulting that the pre-neoplastic lesions were more counted in nano-Fe group than the control or the micro-Fe group.

Lipid peroxidation in serum and liver was evaluated by the TBARS assay. The TBARS value results from lipid peroxidation of polyunsaturated fatty acids and is a marker for oxidative stress. In serum, TBARS value was only increased in nano-Fe group compared with the control group. In liver, TBARS value was also significantly increased in micro-Fe and nano-Fe group compared with the control group. Nelson et al. reported that ferritin levels in serum were positively associated with the presence of adenoma of the colon [12]. Younes et al. reported that oxygen activation and lipid peroxidation might be involved in colon tumorigenesis and cell proliferation [13]. It can be thought that the promotion of lipid peroxidation by nano-Fe may be associated with an increased number of ACF and AC in the colon compared with the control or the micro-Fe group.

Iron store in the liver was measured by ICP-AES method. When the administrated iron is absorbed, the amount of iron deposits in liver is increased. Therefore, the amount of iron deposits in liver is associated with the degree of absorption of the administrated iron. In the present study, micro-Fe and nano-Fe group had higher deposits of iron in the liver of mice than the control group. This result explains that additionally treated micro-Fe and nano-Fe were absorbed well and they might affect to induce the pre-neoplastic lesions in the colonic mucosa.

The additional administration of micro-Fe and nano-Fe promoted the lipid peroxidation in the liver and increased iron deposits in the liver compared with the control group. In conclusion, micro-Fe and nano-Fe were associated with formation of the colonic pre-neoplastic lesions and especially nano-Fe had greatly influenced on generating the pre-neoplastic lesions in colon. The data of this study showed a potentially deleterious effect of intake of nano-Fe as new foods and medicines. More studies may be needed to reveal the relationship between the risk of colon cancer and the various particle sizes of iron.

Acknowledgements

This work was supported by the intramural research grant of Chungbuk National University in 2014.

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저널명이 맞는지 확인해주세요.